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INVITED RESEARCH HIGHLIGHT

Prostate Cancer

Trading in your spindles for blebs: the amoeboid tumor cell phenotype in prostate cancer

Samantha Morley^{1,2,3}, Martin H Hager^{2,3}, Sara G Pollan¹, Beatrice Knudsen¹, Dolores Di Vizio^{1,2,3}, Michael R Freeman^{1,2,3,4}

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Prostate cancer (PCa) remains a principal cause of mortality in developed countries. Because no clinical interventions overcome resistance to androgen ablation therapy, management of castration resistance and metastatic disease remains largely untreatable. Metastasis is a multistep process in which tumor cells lose cell-cell contacts, egress from the primary tumor, intravasate, survive shear stress within the vasculature and extravasate into tissues to colonize ectopic sites. Tumor cells reestablish migratory behaviors employed during nonneoplastic processes such as embryonic development, leukocyte trafficking and wound healing. While mesenchymal motility is an established paradigm of dissemination, an alternate, ‘amoeboid’ phenotype is increasingly appreciated as relevant to human cancer. Here we discuss characteristics and pathways underlying the phenotype, and highlight our findings that the cytoskeletal regulator DIAPH3 governs the mesenchymal-amoeboid transition. We also describe our identification of a new class of tumor-derived microvesicles, large oncosomes, produced by amoeboid cells and with potential clinical utility in prostate and other cancers.

THE AMOEBOID PHENOTYPE

The classic mesenchymal mode of tumor cell migration has been actively investigated

for many years.¹ Cells migrating in this manner polymerize actin into filopodia and lamellipodia at their leading edge, where these protrusions facilitate recognition of chemotactic gradients and adhesion to underlying substrata.^{2,3} At sites of integrin engagement with the extracellular matrix (ECM), focal contacts form and mature into focal adhesions through recruitment and concentration of kinases (e.g., focal adhesion kinase), adaptor proteins (e.g., α -actinin) and actin binding proteins (e.g., paxillin). Following adhesion, actomyosin contractility generates traction forces enabling forward translocation of the cell body. Adhesions at the trailing edge are then released, and the process continues in a cyclic fashion as the cell migrates away from the primary tumor.²

Transition from epithelial to mesenchymal features (EMT) in cancer cells is a well-recognized mechanism of motility that is relevant to disease progression. A defining characteristic of cells having undergone EMT is dependence on pericellular proteolysis for migration. The observation that some migrating cells are relatively insensitive to protease inhibition led to the suggestion that alternative, nonproteolytic mechanisms of tumor cell escape must exist. Wolf *et al.*⁴ were the first to show that, under conditions of protease inhibition, certain tumor cells undergo a dramatic morphological, biochemical and migratory transition, converting from spindle-like to rounded morphologies, with loss of focal integrins. These tumor cells also formed constriction rings, enabling squeezing of the cell body through ECM fibers. Notably, the transition was accomplished in the absence of proteolytic matrix remodeling. Sahai and Marshall extended these observations, showing that RhoA/ROCK (Rho kinase) signaling and pliable matrices prompted tumor cells to adopt

a rounded, blebbing morphology.⁵ Similarly to observations by Wolf and colleagues⁴, inhibition of proteolysis provoked elongated cells to become round, a conversion that induced sensitivity to ROCK inhibitors. Given the migratory resemblance to the amoeba *Dictyostelium discoideum*, this phenotype has been coined ‘amoeboid’ motility.

Following these two seminal studies, further investigation unveiled additional biochemical and cellular features inherent in amoeboid migration. Wilkinson *et al.*⁶ observed that heightened RhoA/ROCK signaling induced cortical actomyosin contractility through phosphorylation of myosin light chain (MLC2). *In vivo*, MLC2 localized perpendicularly to the direction of migration, providing amoeboid cells with the force necessary to deform proximal matrices and thereby push fibers from their path.⁷ ECM deformation appears to confer a migratory advantage, as amoeboid behavior predominates at tumor margins,^{8,9} with migration rates *in vivo* 10–30 times those observed in culture and relative to cells migrating in a mesenchymal fashion.² Amoeboid behavior also confers greater sensitivity to chemotactic agents,^{10,11} potentiating intravasation,^{12,13} and enables cell survival during extensive shear stress within the vasculature.¹⁴ Extravasation and colonization are also promoted, as evinced by increased pulmonary metastases of amoeboid variants in murine metastasis models.^{8,12,15,16} Collectively, these observations suggest that a ‘mesenchymal-to-amoeboid’ transition (MAT) increases tumor cell aggressiveness relative to EMT, and thereby augments transit through the metastatic cascade.¹⁷

RELATIONSHIP OF EMT TO MAT

Both EMT and MAT are adaptive and reversible mechanisms mediating diverse aspects of the

¹Division of Cancer Biology and Therapeutics, Departments of Surgery, Medicine and Biomedical Sciences, and The Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, CA; ²Urological Diseases Research Center, Boston Children's Hospital, Boston, MA; ³Department of Surgery, Harvard Medical School, Boston, MA; ⁴Department of Medicine, David Geffen School of Medicine, University of California, Los Angeles, CA, USA.

Correspondence: Prof. MR Freeman (Michael.Freeman@cshs.org) or Prof. D Di Vizio (Dolores.Divizio@cshs.org)

plasticity underlying dissemination. Not surprisingly these behaviors share promigratory features, however, they are also functionally and morphologically distinguishable.

Intercellular adhesion

Acquisition of a mesenchymal phenotype is characterized by loss of epithelial markers (e.g., E-cadherin) and expression of ectopic markers (e.g. N-cadherin). This 'cadherin switch' weakens adherens junction strength¹, facilitating single-cell migration. Cells migrating in an amoeboid fashion may similarly become extricated from cell-cell constraints through reduced expression of junctional components.¹⁸

Cell-matrix adhesions

A key difference between amoeboid and mesenchymal migration modes lies in their relative dependence on integrins. Cells exhibiting a mesenchymal phenotype display focal, clustered localization and increased utilization of integrins, and differences in integrin subtypes in the front and rear of the cells. These changes strengthen anchorage to the substratum for forward translocation of the cell body.¹⁹ In contrast, amoeboid cells exhibit reduced surface expression and engagement of integrins^{4,20} and display uropods.^{21,22} MAT is associated with reduced focal adhesion kinase autophosphorylation at Y397^{20,23,24}, can be induced by low integrin expression, integrin blocking antibodies or integrin peptidomimetics,²⁵ and is modulated by integrin turnover and downstream signaling.^{26–30} Because an inverse correlation exists between adhesion strength and migration speed, the weaker adhesions of amoeboid cells likely contribute to their faster migration rates.^{25,31}

Pericellular proteolysis

A defining characteristic of EMT is dependence on proteases for 'path generation' during migration. Co-clustering of $\beta 1$ -integrins and matrix metalloproteinases (MMPs)⁴ at sites of ECM contact allows focalized proteolysis of matrix fibers that allosterically impede migration.¹ In contrast, MMPs in amoeboid cells are localized to the cytosol.⁴ Consistent with this distribution, MMPs are less central to amoeboid motility, which can be refractory to protease inhibition.^{4,5,7} MAT thus represents a mechanism by which tumor cells can migrate and invade under conditions where protease activity is suppressed.^{4,32}

Directional and random migratory modes

In three-dimensional (3D) culture, both mesenchymal and amoeboid cells migrate directionally. This directionality is dictated by

the leading edge in mesenchymal cells, yet in amoeboid cells is instead dependent on the site and direction of bleb outgrowth. Such directional migratory behaviors in 3D are in contrast to those in 2D, in which persistent migration in one direction is characteristic only of cells migrating in a mesenchymal fashion. In contrast to directional persistence in 3D, in 2D cultures amoeboid cells instead display random and faster rates of motility,²⁵ consistent with the inverse correlation between directional persistence and migration speed.³¹

Morphogenesis through cytoskeletal remodeling

Morphology constitutes a fundamental distinction between amoeboid and mesenchymal phenotypes. Mesenchymal cells are characteristically elongated and polarized, with leading and trailing edges. In contrast, amoeboid cells are rounded/ellipsoid without signs of planar polarization and display prominent membrane blebs. Actin remodeling is employed by both cell types to produce these respective morphologies. Motile cells operating in a mesenchymal mode use actin polymerization to generate chemo- and substrate-sensing filopodia, and lamellipodia and stress fibers for traction and maturation of focal adhesions.^{3,33} In contrast, amoeboid cells display heightened actomyosin contractility, which provides the force for displacement of matrix fibers^{3,7,33,34} and facilitates bleb dynamics.³⁵ Both mesenchymal and amoeboid cells are naturally occurring subpopulations within the DU145 PCa cell line (**Figure 1a**).

Though less characterized than actin contractility, microtubule (MT) dynamics also influence EMT and MAT. These long, cylindrical tubulin polymers undergo stochastic cycles of elongation and disassembly.³⁶ This 'dynamic instability' underlies MT-dependent processes, perturbation of which elicits numerous features of neoplastic transformation. EMT appears to render the MT cytoskeleton less stable than in differentiated epithelia.³⁷ Functional loss of tumor suppressor proteins can cause MT disruption, which cooperates with the pleiotropic events underlying EMT.³⁷ Regulators of MT dynamics also affect EMT.^{38–40} While relevant to EMT, MT instability may contribute even more substantially to MAT. Inhibiting MT polymerization with vincristine promotes amoeboid invasion through hyperactivation of GEFH1, an activator of RhoA/ROCK⁴¹. Overexpression,⁴² downregulation of inhibitory phosphorylation,⁴² or loss of sequestration by p27kip1,^{12,43} of the MT depolymerizer stathmin also induces MAT,

in part through disruption of endocytic trafficking.⁴⁴ Such events are detected in human tumors,⁴² including PCa.^{45,46}

Consistent with a role for the cytoskeleton in triggering amoeboid behavior, our laboratory has identified the diaphanous-related formin-3, DIAPH3, as potentially a pivotal regulator of MAT in prostate cancer and possibly other tumor types.²³ DIAPH3 belongs to the formin family that shares tandem FH1 and FH2 domains, which nucleate, elongate and bundle linear actin filaments and/or stabilize MT.⁴⁷ DIAPH3 silencing causes redistribution of actin structures (stress fiber loss and cortical MLC2 relocalization) and reduces MT stability, alterations associated with transition to an ellipsoid, blebbing and amoeboid phenotype²³ (**Figure 1b**). In 3D matrices, invading DIAPH3-silenced cells assume rounded morphologies, while controls are elongated. Consistent with these cytoskeletal defects and the amoeboid characteristics above, DIAPH3 silencing disrupts endocytic trafficking, suppresses focal adhesions and promotes migration, invasion and metastatic colonization.²³ Enforced DIAPH3 expression instead induces mesenchymal characteristics, including N-cadherin upregulation, suppression of membrane blebbing and increased stress fiber formation,²³ phenotypes modulated by the phosphorylation state of DIAPH3 at S624. Our studies situate DIAPH3 as a node controlling mesenchymal and amoeboid behaviors.

Non-apoptotic membrane blebbing

While mesenchymal cells display lamellipodia and filopodial protrusions, a defining characteristic of amoeboid cells is protrusion of bulky, non-apoptotic and dynamic membrane blebs from the cell surface. Cortical tension,⁴⁸ substrate adhesion strength,⁴⁹ and relative RhoA and Rac1 activities² influence the choice to bleb or form lamellipodia.⁵⁰ Dissociation of the actin cortex and plasma membrane or alternatively cortical ruptures,^{35,51} initiates bleb formation. Hydrostatic pressure inflates these structures, whose growth is reverted by cortex regeneration.⁵² Blebs contribute to amoeboid migration, and their release can modify the tumor microenvironment (TME, see below).

Our group recently demonstrated that membrane blebs formed from amoeboid cells can be shed into the extracellular space.^{53,54} Such extracellular vesicle (EV) shedding produces atypically large (1–10 μ m) EV that can condition the TME and reach the circulation. We named this type of poorly-characterized particle a 'large oncosome', employing the term 'oncosome' used previously by Janusz Rak and

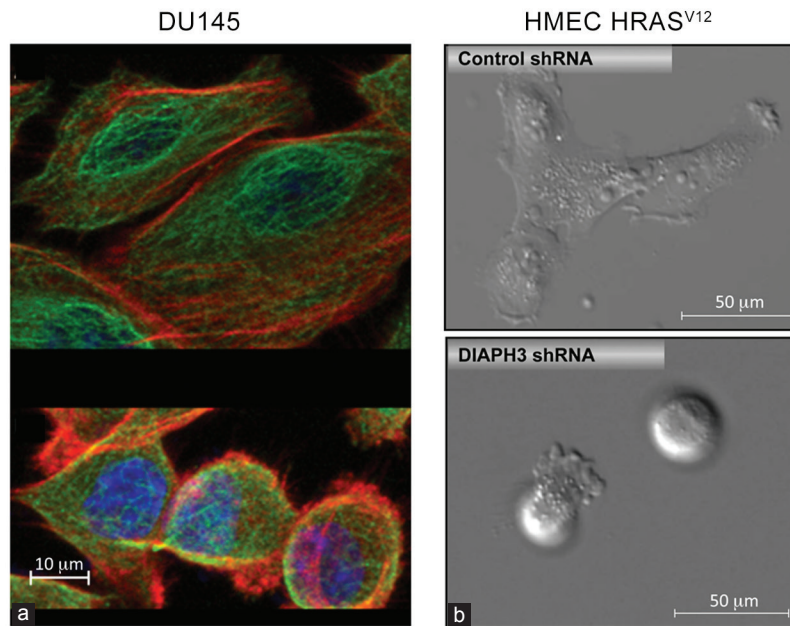


Figure 1: (a) Mesenchymal (top) and amoeboid (bottom) subpopulations occur naturally within DU145 PCa cells. Cells were stained with antitubulin (green) and phalloidin (red) to demonstrate differences in tubulin and actin cytoskeletal organization. The cell nucleus is blue (DAPI). Note the difference in size between mesenchymal and amoeboid cells. (b) Mesenchymal (top)-amoeboid (bottom) transition in HMEC-HRASV12-transformed HMECs upon DIAPH3 silencing.²³ DAPI: 4',6-diamidino-2-phenylindole; HMEC: human mammary epithelial cells; PCa: prostate cancer.

colleagues as a tumor-derived microvesicle that carries tumor biomarkers and can transfer signaling complexes to recipient cells.⁵⁵ Oncosomes horizontally transfer proteins, mRNA, miRNA and metabolites to neighboring tumor and stromal cells,⁵⁵ in a manner distinct from paracrine signaling by soluble factors. The realization that the size range of large oncosomes far exceeds that of other EV (e.g., exosomes, ≤ 70 nm) identified a significant advantage for their visualization and isolation. Thus, while detection of smaller EV requires electron microscopy, large oncosomes are of sufficient size to be visualized by light microscopy or immunofluorescence methods. These tools can be applied in human tissues, raising the possibility for detection of amoeboid cells *in situ*, a significant advance for clinicopathologic detection of this malignant phenotype in tumor biopsies.⁵⁴ In addition to detection techniques in tissues, we have also developed a method for large oncosome identification using size beads and immunoflow cytometry,⁵⁴ and more recently, a filtration-based system enabling exclusion of smaller EV and selective enrichment for large oncosomes.⁵⁶ With these approaches, we have identified large oncosomes shed from cultured cells and in biological fluids from mice and humans with PCa.

Using a quantitative blebbing assay, we observed that the oncogene Akt1 and mitogens of the EGF family promote large

oncosome genesis, a process attenuated by epidermal growth factor receptor (EGFR) inhibition with gefitinib.⁵³ Proteomic analysis of microvesicles shed from cells overexpressing activated Akt1 revealed the presence of numerous signaling mediators, including Akt1, Src and a biomarker for metastatic PCa, caveolin-1.⁵³ More recently, analyses of large oncosomes from tumorigenic RWPE-2 or non-tumorigenic RWPE-1 isogenic prostate cells revealed abundant, differentially-expressed miRNAs.⁵⁶ Large oncosomes display gelatinase activity,⁵³ promote gene expression in recipient fibroblasts⁵⁴ and stimulate migration in cancer and endothelial cells.⁵⁴ Together, these disparate bioactivities suggest that large oncosomes contribute to cancer progression. In support of this notion and MAT induction by DIAPH3 loss,²³ DIAPH3 silencing stimulates formation and shedding of large oncosomes,^{23,53,54} which promote proliferation and motility in recipient cells.⁵³ Similarly, DIAPH3 silencing enhances the secretion of smaller EV, which stimulate proliferation in recipient prostate and heterologous cancer cells (Kim *et al.* in press). Our data suggest that EV secretion is upregulated in amoeboid cells, and that the diverse biological activities of these microvesicles contribute to the malignancy and greater invasiveness of such tumor cell variants.

PATHWAYS MEDIATING THE AMOEBOID PHENOTYPE

Networks underlying MAT remain poorly defined, though their elucidation would greatly facilitate therapeutic strategies with which to impact metastasis. Below, we summarize pathways modulating this transition (Figure 2).

Rho family GTPases, their regulators and downstream targets

The canonical GTPases Rac1, Cdc42 and RhoA, play critical roles in specifying migration strategies.^{5,8,57} Like all GTPases, the Rho family is cyclically activated by guanine nucleotide exchange factors (GEFs) and deactivated by GTPase-activating proteins (GAPs).⁵⁸ RhoA and Rac1 control the transition between amoeboid and mesenchymal phenotypes, respectively, and display an inter-relationship also with Cdc42. While inducing filopodia during mesenchymal motility, Cdc42 also paradoxically promotes amoeboid migration; activation by DOCK10 induces MAT,⁵⁹ while this Cdc42-mediated transition is suppressed by RasGRF1/2.¹⁵ In contrast to Cdc42, Rac1 functions as an antagonist of the amoeboid phenotype. Hyperactivation of Rac1 by the GEF DOCK3 promotes mesenchymal behavior,⁸ while inactivation by the GAPs ARHGGEF22⁸ or FilGAP⁶⁰ promotes MAT. Arguably, the most characterized GTPase mediator of the amoeboid transition is RhoA, which is activated by disparate upstream GEFs.^{27,61–64} Signaling through its effector kinase ROCK, RhoA promotes actomyosin contractility, and thereby enables ECM deformation and 'path finding' through tissue matrices. RhoA/ROCK signaling is positively regulated by PDK1¹⁶ and p53 loss,³³ and negatively regulated by RhoE/Rnd3^{16,65} and the ubiquitin E3 ligase SMURF1.³⁴ Consistent with the requirement of RhoA/ROCK for MAT, we observed that DIAPH3 silencing promotes ROCK activation, as evinced by enhanced MLC2 and MYPT-1 phosphorylation.²³

Receptor tyrosine kinases

Chemotactic sensitivity is heightened by transition to an amoeboid phenotype. In agreement with their contribution to PCa progression and metastasis,^{66,67} the hepatocyte growth factor receptor, c-MET,⁶⁸ and Ephrin A1 receptor, EphA2,^{69,70} are both implicated in MAT. Extensive work by the Condeelis laboratory demonstrates that amoeboid migration is also highly EGFR-responsive, with amoeboid cells hypersensitized to EGF, a soluble factor that facilitates chemotaxis and promotes

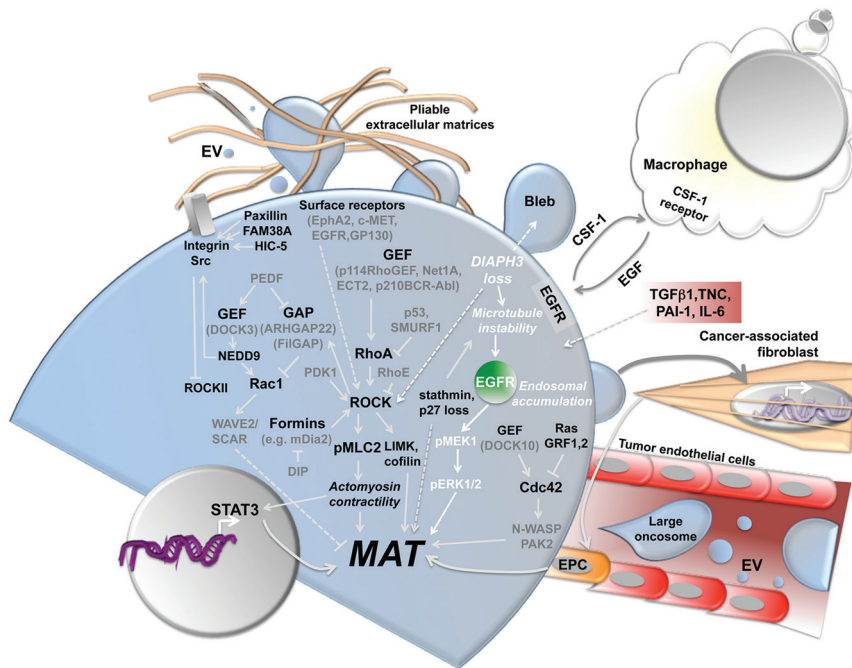


Figure 2: Signal transduction mechanisms and cell processes that regulate or are associated with the amoeboid phenotype. CSF-1: Colony stimulating factor 1; EGFR: epidermal growth factor receptor; EPC: endothelial progenitor cell; EV: extracellular vesicle; GAP: GTPase-activating protein; GEF: guanine nucleotide exchange factor; IL-6: interleukin-6; MAT: mesenchymal-to-amoeboid transition; PAI: plasminogen activator inhibitor type-1; TGFβ1: transforming growth factor beta 1; TNC: Tenascin C.

metastasis.^{14,71} Concordantly, we observed elevated EGFR activation and EGF-responsive membrane blebbing, migration and invasion in *DIAPH3*-deficient PCa cells.^{23,53}

The TME

Mitogenic and morphogenic components within the TME participate in conversion of tumor cells to an amoeboid phenotype. Secretion of tenascin C⁷² or plasminogen activator inhibitor type-1 (PAI-1)⁷³ by tumor cells promotes MAT, a transition similarly induced by the proinflammatory cytokines IL-6⁷⁴ and transforming growth factor β1.⁶² Crosstalk with stroma also potentiates amoeboid migration.⁷⁵ Paracrine interactions, through reciprocal ligand secretion and complementary receptor expression, promote co-migration of amoeboid cells and macrophages.¹³ Carcinoma cell migration is further mediated by activation of cancer-associated fibroblasts (CAFs) by the pro-inflammatory cytokine OSM, which promotes CAF contractility and in turn ECM remodeling.⁷⁴ While CAFs induce EMT in PCa cells, in cooperation with the tumor cells they recruit endothelial progenitor cells that stimulate further transition to an amoeboid phenotype.⁷⁶ Adhesion of PCa cells to endothelial progenitor cells increases following MAT, potentially promoting neovascularization and intravasation.⁷⁶

Amoeboid-derived large oncosomes have the potential to pleiotropically condition the TME. Enriched in numerous oncogenic biomolecules, large oncosomes stimulate signal transduction, proliferation and migration in recipient tumor cells,^{53,54} and gene expression in CAFs.⁵⁶ Large oncosomes upregulate pro-metastatic factors in fibroblasts, enhance migration in tumor endothelial cells and activate stromal myofibroblasts to enhance PCa cell migration.^{53,54} EV shed by amoeboid cells also suppress immune cell proliferation (Kim *et al.* in press). It is tempting to speculate that amoeboid-derived EV contribute to the 'relay system' proposed by Wyckoff and colleagues,¹³ by transmitting promigratory signals from the minority of highly chemotactic amoeboid cells to the remainder of the tumor mass.

Cytoskeletal remodeling and formins

Cytoskeletal rearrangements are fundamental to the reprogramming underlying motile behaviors in tumor cells. Alterations in actin and MT networks thus contribute to both EMT and MAT. Formins regulate cytoskeletal dynamics downstream of Rho GTPases⁷⁷ and are thereby poised to contribute to both mesenchymal and amoeboid morphogenesis. Indeed, several formins and their upstream regulators induce transition to an amoeboid phenotype. Overexpression

of the Formin-like protein 2 evokes MAT,⁷⁸ and FHOD1 promotes membrane blebbing in conjunction with ROCK1 and Src⁷⁹, while Dia1 promotes bleb-based cell motility through the GEF LARG.⁸⁰ Eisenmann and colleagues demonstrated that inhibition of mDia2, through binding to DIA-interacting protein (DIP), similarly induces an amoeboid transition.^{81,82} Interestingly, the *DIAPH3* locus encodes multiple splice variants that differentially impact actin dynamics and membrane blebbing.⁸³ Thus, formins both positively and negatively participate in the amoeboid transition.

Genomic deletion of the formin *DIAPH3*

Our laboratory has demonstrated a relationship between *DIAPH3* and the amoeboid phenotype in PCa.²³ We identified the *DIAPH3* locus within a small frequent focal deletion on chromosome 13q. Copy number variation analyses revealed a significant correlation between *DIAPH3* loss and disease progression. Genomic *DIAPH3* deletions increased in frequency with Gleason grade, most frequent in metastatic PCa.²³ A 100K SNP array analysis of primary and metastatic tumors yielded a similar conclusion, one supported by fluorescent *in situ* hybridization on an independent cohort.⁵³ In tissues from PCa patients, *DIAPH3* protein levels were diminished in metastases relative to primary tumor or benign prostate epithelia. *DIAPH3* loss was also more prevalent in disseminated tumor cells from patients with advanced disease, relative to those with organ-confined disease or solid tumors.²³

In agreement with association with metastasis, targeting *DIAPH3* by RNAi in cultured cells evoked an amoeboid transition, characterized by membrane blebbing, cell rounding, hyperactivation of ROCK/MLC2 and fast, random migration²³. Echoing the above examples of enhanced extravasation/colonization of amoeboid cells,^{8,12,15,16} *DIAPH3* silencing potentiated metastases in mice. Molecularly, *DIAPH3* loss destabilized MT, as evinced by diminished tubulin acetylation, a posttranslational modification accumulating on MT with slow turnover rates (stable MT).⁸⁴ The cytoskeletal defect was associated with disrupted endocytic trafficking of EGFR,²³ whose activation promotes blebbing in PCa cells.⁵³ EGFR accumulated in early endosomes, with transport to lysosomes and membrane recycling both mitigated. Consequently, attenuation of receptor activity was delayed, evoking downstream ERK1/2 hyperactivation. Sustained MEK1/ERK1/2 signaling was essential for maintenance of MAT. These

findings suggest that assessing *DIAPH3* lesions in PCa patients may have prognostic utility.

CONCLUSIONS

The studies described in this article highlight the importance of tumor cell plasticity in patient stratification and attempts to discover new, clinically informative biomarkers. Methods for identifying amoeboid cells in human tumors are emerging, including detection of large oncosome features in tumor tissue or in the blood. The role of the amoeboid phenotype in tumor progression is still poorly understood and much fascinating tumor biology lies ahead, waiting discovery.

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Nothing to declare.

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